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Dual Role of Desferrioxamine in *Erwinia amylovora* Pathogenicity

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To investigate the role of iron in *Erwinia amylovora* pathogenicity, virulence properties of two mutants of strain CFBP 1430 isolated by insertional mutagenesis and affected in the iron transport pathway mediated by desferrioxamine (DFO) were analyzed. One mutation (*dfoA::*Mu*d*IIpR13) disrupts DFO biosynthesis. The present analysis shows that this mutation affects an open reading frame that belongs to a biosynthetic gene cluster and shares identity with the *alcA* gene required for synthesis of the siderophore alcaligin in *Bordetella* spp. A second mutation (*foxR::*Mu*d*IIpR13) affects the synthesis of the ferrioxamine receptor FoxR, encoded by the *foxR* gene, and was shown to be transcribed into a monocistronic message. Accordingly, the *foxR* mutant accumulates DFO in the external medium. The growth of the mutants when supplied with various iron sources was examined; it indicates that the production of DFO and the specific transport of the DFO ferric complex are required only when iron is strongly liganded. Pathogenicity was scored after inoculation of apple seedlings and after infection of apple flowers. On seedlings, the DFO biosynthetic mutant behaved like the wild-type strain while the frequency of necrotic plants caused by the receptor mutant decreased by a factor of two to five, depending on the initial inoculum. On flowers, both mutants were strongly affected in their ability to initiate a necrotic symptom and their growth was reduced by two orders of magnitude relative to the wild-type strain. However, the virulence of the *dfoA* mutant varied with the inoculum concentration. Unlike the *foxR* mutant, the *dfoA* mutant only weakly induced plant cell electrolyte leakage in tobacco leaf disks. The supply with exogenous DFO, only when iron free, restored the ability to induce electrolyte leakage to the *dfoA* mutant and increased the leakage induced by other strains. DFO alone was not an inducer. Iron-free DFO was able to protect *E. amylovora* cells against lethal doses of hydrogen peroxide. The main conclusion was that production of DFO in *E. amylovora* during pathogenesis is not only a critical function for iron acquisition, but can play a role in the oxidative burst elicited by the bacteria.

Additional keywords: fire blight.

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Erwinia amylovora is an enterobacterium that causes fire blight, a disease of members of the Maloideae characterized by a progressive necrosis of tissues of infected aerial parts of the plant, often associated with ooze production. Natural infections mainly occur through wounds on succulent shoots and young leaves, as well as through natural openings, especially on flowers (Thomson 1986). On susceptible hosts, bacteria first move through the intercellular spaces of parenchyma and in a later stage in the xylem vessels, thus provoking extensive lesions, and sometimes complete dieback of the tree, under favorable climatic conditions. The diseased parts of the plant become brown or black as if they had been swept by fire. Two main genetic determinants have been shown to be involved in *E. amylovora* pathogenicity. Disruption by gene mutation of the biosynthesis of amylovoran, an extracellular polysaccharide encoded by the *ams* genes, results in loss of virulence (Belleman and Geider 1992; Bernhard et al. 1990; Bugert and Geider 1995). The *hrp-dsp* genes clustered within a 40-kb genomic region encode functions responsible for the secretion of protein factors involved in symptom development on susceptible hosts and the hypersensitive response (HR) on nonhosts (Steinberger and Beer 1988; Barny et al. 1990; Walter et al. 1990; Bauer and Beer 1991; Wei and Beer 1993; Barny 1995; Bogdanove et al. 1996; Gaudriault et al. 1997). The *hrp* cluster is involved in electrolyte leakage induction from plant cells in both disease and HRs, whereas the *dsp* region is essential for electrolyte leakage in the compatible situation only (Brisset and Paulin 1991, 1992). However, a faster efflux was observed in the incompatible situation.

During the infection process the pathogen must face several obstacles, one of which, low iron availability, may create a situation of competition between host and pathogen. Indeed, iron is essential for almost all forms of life, because it acts as a catalyst of crucial metabolic processes. In living tissues, this metal is complexed to specific molecules, enhancing its solubilization and assimilation. High-affinity iron chelating pro-

teins, such as transferrins present in vertebrate body fluids, may produce a bacteriostatic effect by deprivation of nutritional iron. Microorganisms may circumvent this difficulty by excreting siderophores that, due to their high affinity for the ferric ion, can remove iron from a variety of available mineral and organic substrates. High-affinity iron acquisition systems are virulence factors for many animal-pathogenic bacteria (Bullen et al. 1991; Payne 1993; Weinberg and Weinberg 1995). The role of iron in plant host-pathogen interactions has been investigated in soft rot caused by *E. chrysanthemi* 3937 on African violets (Enard et al. 1988). In this disease, the low accessibility of iron during pathogenesis acts as a signal triggering the coordinated expression of several virulence factors contributing to iron acquisition, such as a high-affinity iron assimilation system mediated by chrysobactin (Expert et al. 1996).

Under iron-limiting conditions, *E. amylovora* CFBP 1430 produces hydroxamate-type siderophores (Vanneste 1995) characterized as cyclic desferrioxamines (DFOs), mostly DFO E (Feistner et al. 1993; Kachadourian et al. 1996) and the specific receptor FoxR, a 70,000-Da protein needed for the passage of their ferric complexes across the outer membrane (Kachadourian et al. 1996). Among microbial siderophores, DFOs are molecules of special interest. DFO B, widely used since 1962 in the treatment of humans with iron overload and iron poisoning, has also attracted much attention as a laboratory tool for the study of the possible involvement of iron in production of oxy-radicals, mediators of tissue injuries in several human diseases (for review, see Halliwell 1989). Due to its high iron binding constant, DFO inhibits the generation of the highly reactive hydroxyl radical OH° from $O_2^{\circ -}$ and H_2O_2 in the presence of iron ions, and has generally been described as protective against free radical damage in several animal models. But, in vitro, DFO can also react at high concentrations directly with $O_2^{\circ -}$ and H_2O_2 or act as substrate for peroxidase in the presence of H_2O_2, leading in both cases to the generation of a reactive nitroxide radical that might be responsible for toxic side effects sometimes observed in vivo (Davies et al. 1987; Morehouse et al. 1987). As most plant defense reactions against pathogens involve generation of harmful reactive oxygen species (Baker and Orlandi 1995; Low and Merida 1996), it was of particular interest to investigate the role of the DFO-mediated iron uptake system of *E. amylovora* in pathogenesis.

Several iron uptake negative mutants of *E. amylovora* CFBP 1430 isolated by insertional mutagenesis have been recently identified. In mutant VD61, the mutation (*dfo-61*::Mu*d*IIpR13) disrupts the DFO biosynthetic pathway; in VD17, the mutation (*foxR-17*::Mu*d*IIpR13) affects the synthesis of the FoxR receptor and the mutant accumulates DFO in the external medium because of its failure to transport back the DFO ferric complex (Kachadourian et al. 1996). In the present study, the genotype of these mutants was analyzed further. A detailed description is presented of their pathogenic behavior, compared with the wild-type strain, when confronted with compatible or incompatible situations. The data demonstrate that in *E. amylovora* production of DFOs is a critical function for bacterial iron acquisition during pathogenesis, as well as for bacterial induction of electrolyte leakage from plant cells. A dual role of DFO during plant/*E. amylovora* interactions is proposed.

RESULTS

Genetic characterization of *E. amylovora dfo-61* and *foxR-17* mutations.

In the DFO nonproducing mutant VD61, the *dfo-61* mutation results from a single insertion of the Mu*d*IIpR13 genome (Kachadourian et al. 1996). In order to pick up the cognate wild-type gene, we first cloned the host DNA sequence flanking the left terminal part of the Mu prophage. The *Sal*I total DNA digest from the mutant strain was cloned into pUC18. After electroporation of JM109 cells, a few Cm^r, Ap^r transformant clones were recovered and their plasmid content analyzed. The recombinant plasmid pAL7, carrying a host phage DNA insert of 4.8 kb in length, was identified (Fig. 1A). This fragment was used as a DNA-DNA hybridization probe to score the corresponding restriction fragment in the wild-type genomic DNA and to screen a wild-type gene library. A 6-kb *Sal*I fragment was identified. This fragment was found to be present in the insert of approximately 24 kb carried by the cosmid p13A8 detected with the probe (Fig. 1A). This cosmid was mobilized with the helper plasmid pRK2013 into VD61 and the enterobactin-deficient *E. coli* strain, RW193. Tc^r transconjugants were streaked on Chrome Azurol S (CAS)-agar medium. Both types of transconjugants produced a halo and *E. amylovora* transconjugants could grow on ethylenediamine-*N*-*N*′-bis(2-hydroxyphenyl)acetic acid (EDDHA)-agar medium. A bioassay indicated the presence of DFO in low iron cultures of *E. amylovora* and *Escherichia coli* transconjugants. These data indicate that the function missing in mutant VD61 is encoded from a cluster of genes involved in biosynthesis of DFO. The sequence of the Mu*d*IIpR13 insertion site was determined over 900 bp on both strands, with plasmid pAL7 as template and a primer complementary to the left end of Mu (Fig. 2). The Mu DNA was shown to interrupt an open reading frame (ORF), designated as *dfoA*, sharing 58% identity with the *alcA* gene of *Bordetella bronchiseptica*, at a site corresponding to nucleotide 1530 in the *alcA* ORF (Giardina et al. 1995).

The *foxR* gene was previously mapped to a 4-kb *Eco*RI-*Hin*dIII fragment in the genomic insert of the recombinant cosmid pAL2 (Kachadourian et al. 1996; Fig. 1B). Scanning of the nucleotide sequence of this fragment allowed the characterization of a 2,115-bp ORF whose deduced amino acid sequence is 65% identical to that of FoxA, the ferrioxamine receptor of *Yersinia enterocolitica* (Fig. 3). The 2-kb *Eco*RI-*Sal*I fragment from plasmid pAL2, internal to the *foxR* gene (Fig. 1B), was used as a ^{32}P-labeled probe in a Northern (RNA) blot analysis to search for the presence of specific *foxR* gene transcripts. An mRNA transcript approximately 2,100 bases long was detected in total RNA preparations from cultures of the wild-type strain grown under iron-restricted conditions (Fig. 1B). Thus, the *foxR* gene is an iron-repressible, monocistronic unit. The Mu*d*IIpR13 insertion inactivating the *foxR* gene (*foxR-17*) was located to the left relative to the left-hand *Sal*I site (Fig. 1B).

Growth of iron uptake deficient mutants of *E. amylovora* under various conditions in iron.

It was previously shown that the mutant harboring the mutation *dfo-61* was unable to use ferric citrate, the major iron transporter in plants, as an iron source (Kachadourian et al.

1996). This indicated that *E. amylovora* cells do not produce a ferric citrate specific transport system, such as the system identified in *E. coli* (Härle et al. 1995). To further determine the behavior of the mutants in conditions of iron limitation, their growth was compared with that of the wild-type strain, when cultured in Tris medium supplemented with iron chelators such as EDDHA and 2,2′-dipyridyl, or not (Fig. 4). Tris medium contains traces of iron (<1 μM) due to the presence of phosphate. EDDHA specifically chelates ferric ions while 2,2′-dipyridyl is a ferrous ion chelator. Iron replete conditions were provided by the addition of ferric chloride (20 μM). The growth of the wild-type strain was only weakly affected in Tris medium since its doubling time was 4 h instead of 3 h in the presence of $FeCl_3$. The presence of EDDHA (50 μM) increased its doubling time by a factor of 1.9 while its growth was strongly reduced in the presence of 2,2′-dipyridyl (50 μM). The low iron content in Tris medium did not strongly affect the growth of the *dfoA* mutant while the addition of EDDHA or 2,2′-dipyridyl was efficient. The growth of the *foxR* mutant was affected in Tris medium even supplemented with $FeCl_3$, very reduced in the presence of EDDHA, and eliminated in the presence of 2,2′-dipyridyl. The reduced growth rate of the receptor mutant in all conditions relative to the DFO nonproducer was explained by a decrease in ferric iron availability due to the accumulation of DFO in the medium. These data indicate that the DFO-dependent iron uptake system encoded by *E. amylovora* is necessary to overcome the conditions of iron limitation caused by the presence of ferric ion chelators. EDDHA scavenges all traces of ferric ions present in Tris medium, which explains why the growth of the *dfoA* mutant is more severely reduced in the presence than in the absence of the chelator. Furthermore, the fact that 2,2′-dipyridyl strongly reduces not only the growth of the mutants but also that of the wild-type strain suggests the occurrence in *E. amylovora* of a ferrous iron transport system that could be of prime importance in conditions of microaerophilly.

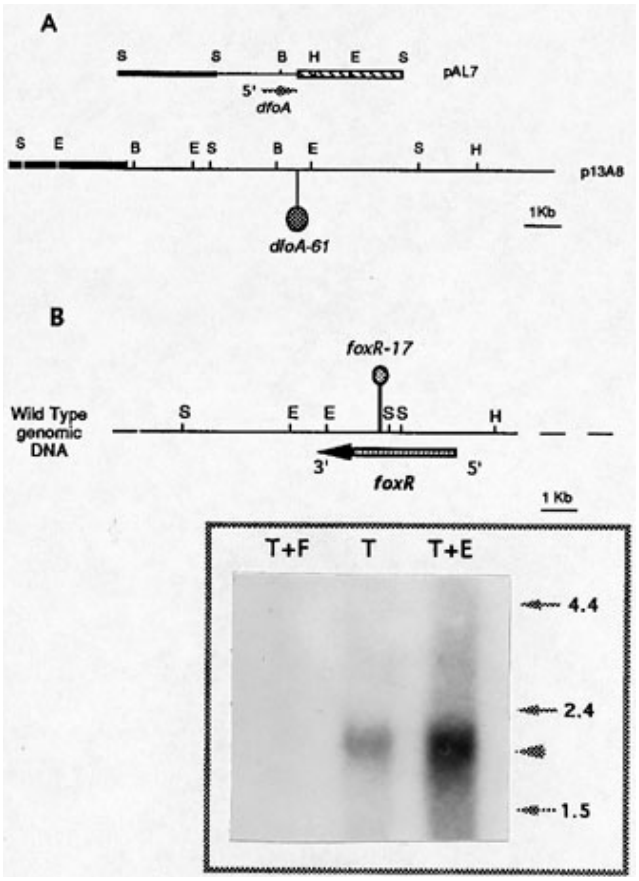

Fig. 1. Characterization of *dfoA-61* and *foxR-17* mutations. Physical maps of *Erwinia amylovora* genomic regions encompassing the mutations are shown. **A,** Hatched bar refers to left part of the Mu*d*IIpR13 prophage responsible for the *dfoA-61* mutation and filled bar to pUC18 and pLA2917 vector DNA, respectively. Predicted transcriptional orientation of the *dfoA* open reading frame is indicated by arrow. **B,** *foxR-17* mutation is shown to disrupt *foxR* gene (indicated by arrow), whose transcriptional activity is visualized in the autoradiogram below. Equal aliquots of RNA (1.25 μg) from wild-type cells grown in T medium supplemented with $FeCl_3$ (lane T+F), ethylene-diamine-*N*-*N*′-bis(2-hydroxyphenyl)acetic acid (EDDHA) (lane T+E) or not (lane T) to an OD_{600} of 0.7 were analyzed by Northern (RNA) blot hybridization, with the *Eco*RI-*Sal*I genomic fragment as a ^{32}P-labeled probe. FoxR mRNA transcripts are indicated by arrowhead. Positions of single-stranded RNA molecular weight markers (in kilobases) are indicated. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

Pathogenic behavior of iron uptake deficient mutants after inoculation of apple seedlings.

The virulence properties of the *dfoA* and *foxR* mutants were first tested after wound inoculation on leaves of apple seedlings. Two concentrations of bacterial cells (10^7 and 10^8 CFU/ml) of the mutants and the wild type were used. After 14 days, a systemic necrosis was apparent on most inoculated plants with the wild-type strain, whatever the initial inoculum (Fig. 5). No plants were found to display only localized symptoms. The *dfoA* mutant behaved like the wild type, while the frequencies of infected plants obtained with the *foxR* mutant were reduced by factors of two and five with initial inocula of 10^8 and 10^7 CFU/ml, respectively. When present, symptoms appeared at the same rate, whatever the strain or mutants. These data are consistent with the existence of a link between an efficient iron uptake system and expression of pathogenicity.

Pathogenic behavior of iron uptake deficient mutants after inoculation of apple flowers.

To analyze the behavior of the mutants in conditions closer to the most common naturally occurring situation (blossom infections), pathogenicity of the mutants was tested on apple flowers. The ability of *E. amylovora* to directly invade the nectaries made it possible to devise a test without wounding during artificial inoculation. For each strain, two concentrations of inoculum, 10^6 and 10^8 CFU/ml, were used. The experiments were conducted during two consecutive springs, in the greenhouse. Both concentrations of the wild type provoked more than 80% blighted flowers. Symptoms appeared 4 days after infection, beginning with blackening of the anthers, followed by necrosis of the calyx. In some cases, no necrotic symptom was apparent, but ooze was abundantly produced. The appearance of blighted flowers was scored during 14 days following infection (Fig. 6A). All symptoms initiated within the first 4 days expanded to the whole flower during the next 3 days. Virulence of the *foxR* mutant was strongly reduced, as no more than 10% of inoculated flowers became blighted whatever the inoculum dose. In contrast, the virulence of the *dfoA* mutant was inoculum dependent, since the percentage of successful inoculations shifted from 70 to 10% as the inocu-

lum decreased by two orders of magnitude. The dynamics of bacterial populations after inoculation were determined (Fig. 6B). The growth of the mutants, compared with the wild-type strain, was reduced by two orders of magnitude, regardless of the initial inoculum. No significant difference between the growth rates of the two mutants was recorded. These results indicate that the iron conditions encountered by bacterial cells in flower tissues were restrictive enough to limit the growth of the two mutants in the same range. Therefore, the pathogenic behavior of the two mutants was not strictly related to their growth rates.

Electrolyte leakage induced by iron uptake deficient mutants in tobacco leaf disks.

To further understand the physiological mechanism underlying reactions induced by the mutants, their ability to induce electrolyte leakage in the incompatible (tobacco) situation was analyzed. Electrolyte leakage was determined by measuring the conductivity of a buffer in which leaf disks were incubated after infiltration of a bacterial suspension. Bacterial cell concentrations in the range of 10^9 CFU/ml always caused a leakage for the wild type and iron uptake deficient mutants (data not shown). The *hrp* strain and buffer used as controls did not cause leakage (Fig. 7). For lower bacterial concentrations (10^8 CFU/ml), the *dfoA* mutant appeared to be significantly reduced, compared with the wild type and *foxR* mutant (Fig. 7A). The addition of DFO B increased the leakage except for controls. The addition of ferrioxamine (ferri-DFO B) reduced the leakage (Fig. 7B). These experiments show that the release of DFO by bacterial cells harboring a functional Hrp system is involved in production of electrolyte leakage by nonhost plant cells.

Protective effect of DFO on bacterial cells exposed to hydrogen peroxide.

As DFO is known to be a protective agent to oxidative cell damage, we investigated the possibility that the failure of the *dfoA* mutant to enhance plant cell electrolyte leakage was due to a decrease in viable counts resulting from antimicrobial activity of reactive oxygen species produced at the onset of infection (Low and Merida 1996). The exposure of *E. amylovora* cells to doses of hydrogen peroxide ranging from 0.1 to 10 mM had a lethal effect and bacterial killing was dose dependent (data not shown). When bacterial cells exposed to 1 mM hydrogen peroxide were supplied with physiological concentrations of DFO B (50 μM), a protective effect against oxidative killing was observed (Table 1). On the other hand, no protection was shown in the presence of ferrioxamine. In all cases, DFO B was more efficient in conferring protection than ferrous iron chelators known to block the Fenton reaction, such as ferrozine and 2,2′-dipyridyl (Table 1). The toxicity at low concentrations of hydrogen peroxide toward *E. coli* cells essentially results from its decomposition into reactive radicals, through three reactions known collectively as the Haber-

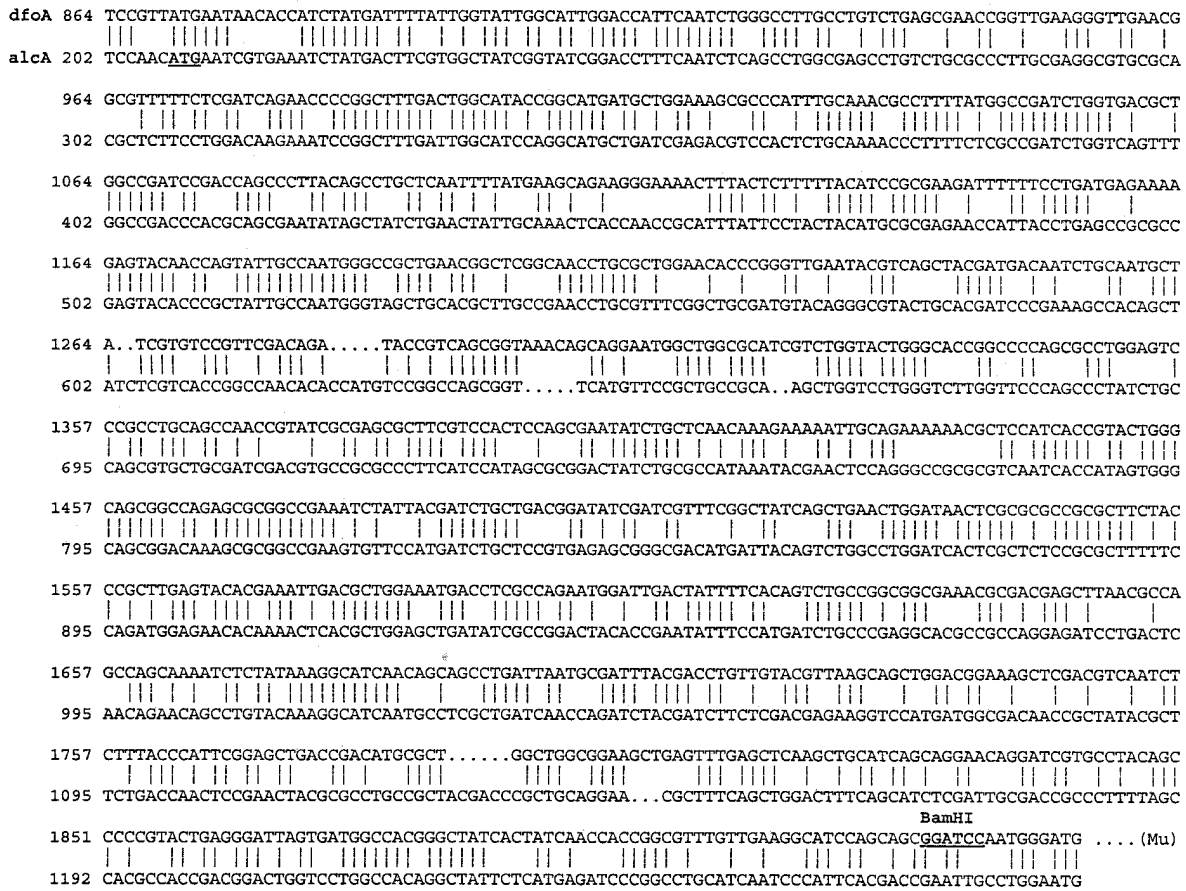

Fig. 2. Nucleotide sequence flanking *dfoA*::Mu*d*IIpR13 mutation. Alignment with *alcA* gene of *Bordetella bronchiseptica* is shown. Details in the text.

Weiss cycle in which the Fe(II)/Fe(III) couple acts as a redox catalyst (Imlay et al. 1988). Thus, it was concluded that DFO through its powerful Fe(III) scavenging activity interrupts the chain reaction accompanying the reduction of hydrogen peroxide.

DISCUSSION

This work was aimed at analyzing the pathogenicity of two mutants of *E. amylovora* altered in their high-affinity iron uptake pathway mediated by DFO. These two mutants, as initially described, contain a single insertion of a mini-Mu prophage affecting the biosynthesis of DFO (*dfo-61*) and that of the ferrioxamine outer membrane receptor (*foxR-17*), respectively. In this report, it is shown that the *dfo-61* insertion inactivates an ORF (*dfoA*) whose predicted translation product shares identity with the *alcA* gene-encoded protein of *Bordetella* spp. In *B. pertussis*, the *alcA* gene belongs to the *alcABC* operon governing the synthesis of the dihydroxamate siderophore alcaligin (Kang et al. 1996). The AlcA product is predicted to be an oxygenase catalyzing the hydroxylation of putrescine in the putative alcaligin biosynthetic pathway. A reaction of N-hydroxylation enabling the conversion of cadaverine to 1-amino-5-(*N-hydroxy*)aminopentane is supposed to take place in the DFO biosynthesis (Feistner 1995). In addition, the *dfoA* ORF was shown to belong to a cluster of genes able to confer on *E. coli* cells the capacity to produce DFO. The organization of the DFO biosynthetic genes remains to be determined. The *foxR-17* insertion disrupts the *foxR* gene, characterized as an iron-regulated, monocistronic, transcriptional unit. The presence of a potential Fur-binding site (5′-AAAAAAAGATAATAATTCTC-3′) overlapping the *foxR* promoter suggests that, in *E. amylovora* as in many bacterial species, iron regulation is achieved by a homologue of the Fur transcriptional repressor (de Lorenzo et al. 1987).

In contrast to other nonpathogenic mutants of *E. amylovora* described so far, including Hrp/Dsp and Ams deficient mutants that fail to produce symptoms on host plants, the mutants affected in DFO-mediated iron uptake are still able to cause fire blight in certain conditions. Indeed, the lack of a func-

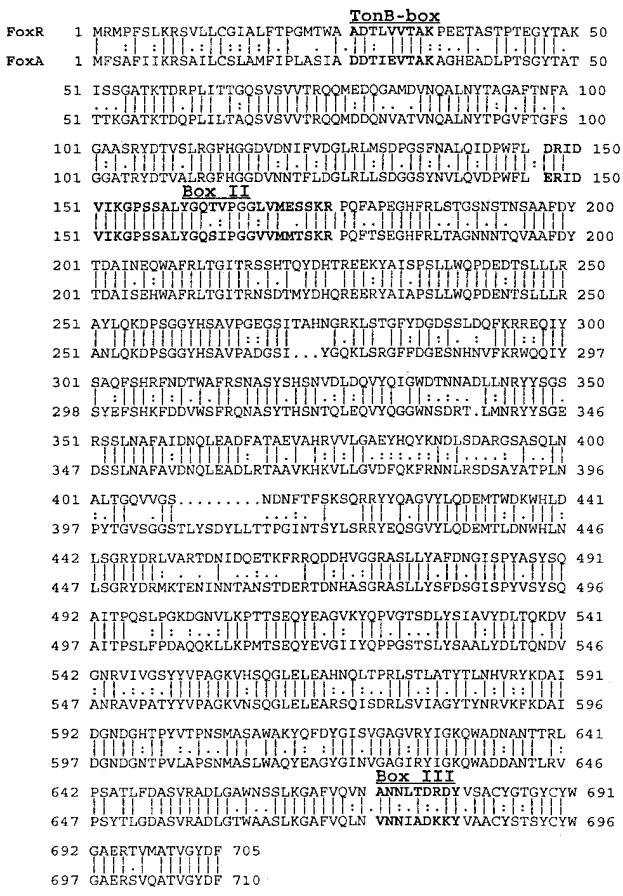

Fig. 3. Alignment of FoxR and FoxA proteins. FoxA (Bäumler and Hantke 1992) and FoxR contain consensus sequences (boldface letters) characteristic of TonB-dependent receptors. Vertical lines indicate identical residues; dots indicate residues with similar chemical properties.

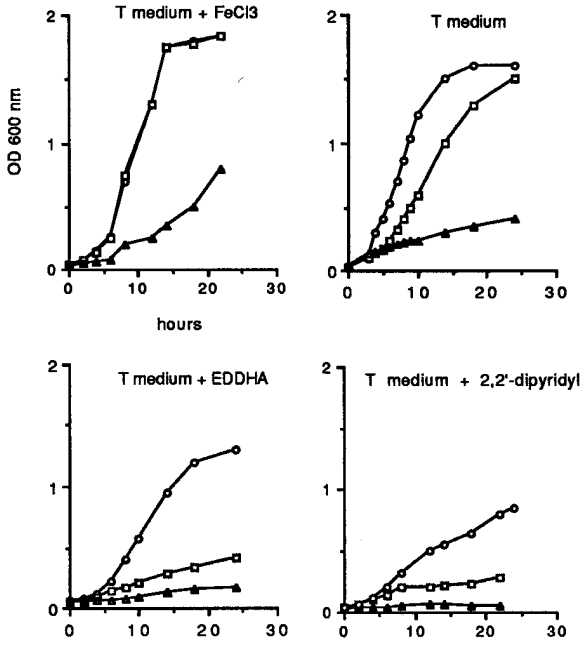

Fig. 4. Growth of wild-type strain (circles) and its derivatives harboring the mutation *dfoA-61* (squares) or *foxR-17* (triangles) in T medium, supplemented with iron or iron chelators as indicated. Details in the text.

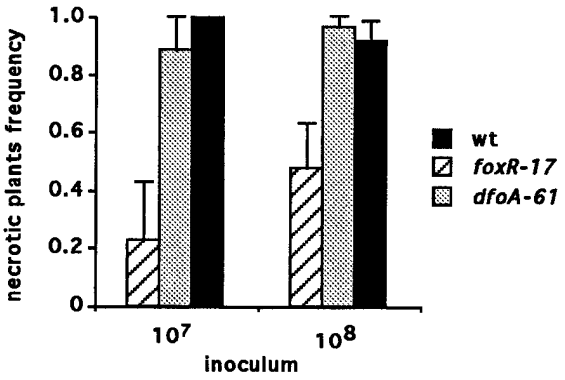

Fig. 5. Pathogenicity of wild-type strain and its derivatives harboring the mutation *dfoA-61* or *foxR-17* on apple seedlings. Details in Material and Methods. Bar heights correspond to mean values obtained for each strain and each inoculum during three independant assays. Standard deviations are indicated.

tional DFO-dependent iron uptake system did not appear to be critical when pathogenicity was tested on seedlings. On the other hand, the test on flowers proved to be discriminative. It shows that iron is a limiting factor for bacterial growth during the infection process. The fact that the response triggered by the two mutants was a reaction of "all or nothing" indicates that iron is critical at the onset of infection. Several reasons may explain the discrepancy in the behavior of the mutants in the two pathogenicity tests. In the test on flowers, the entry of bacterial cells takes place through natural openings, whereas the inoculation on seedlings involves wounding, which could supply bacterial cells with iron, giving them the fitness necessary for successful infection of the plant. Also, the flower and the leaf are different organs with tissues at different physiological stages, implying a mobilization of iron that may be also different. For instance, the occurrence of an active de novo synthesis of ferritin in buds has been reported (Briat et al. 1995).

Otherwise, the difference of behavior between the two mutants in pathogenicity is noteworthy. On flowers, the response induced by the *dfoA* mutant is dependent on the concentration of inoculum, suggesting that the growth defect of the mutant could be overcome by a critical bacterial mass. In this case, the multiplication of bacteria would depend on a number of dead cells able to release enough iron to fulfill the needs of growing cells. In contrast, the behavior of the *foxR* mutant did not change in relation to the initial inoculum, and the lack of aggressiveness of this mutant could be related to its potential to accumulate DFO in the environment, thus trapping any potentially available iron. In addition, the fact that this mutant continually excretes its siderophore may reduce its fitness in planta. However, both mutants grew on flowers at the same rate, which suggests that DFO could play an additional role. In this respect the analysis of electrolyte leakage caused by the mutants is informative. It indicates that bacterial cells with a functional *hrp* region create physiological conditions prone

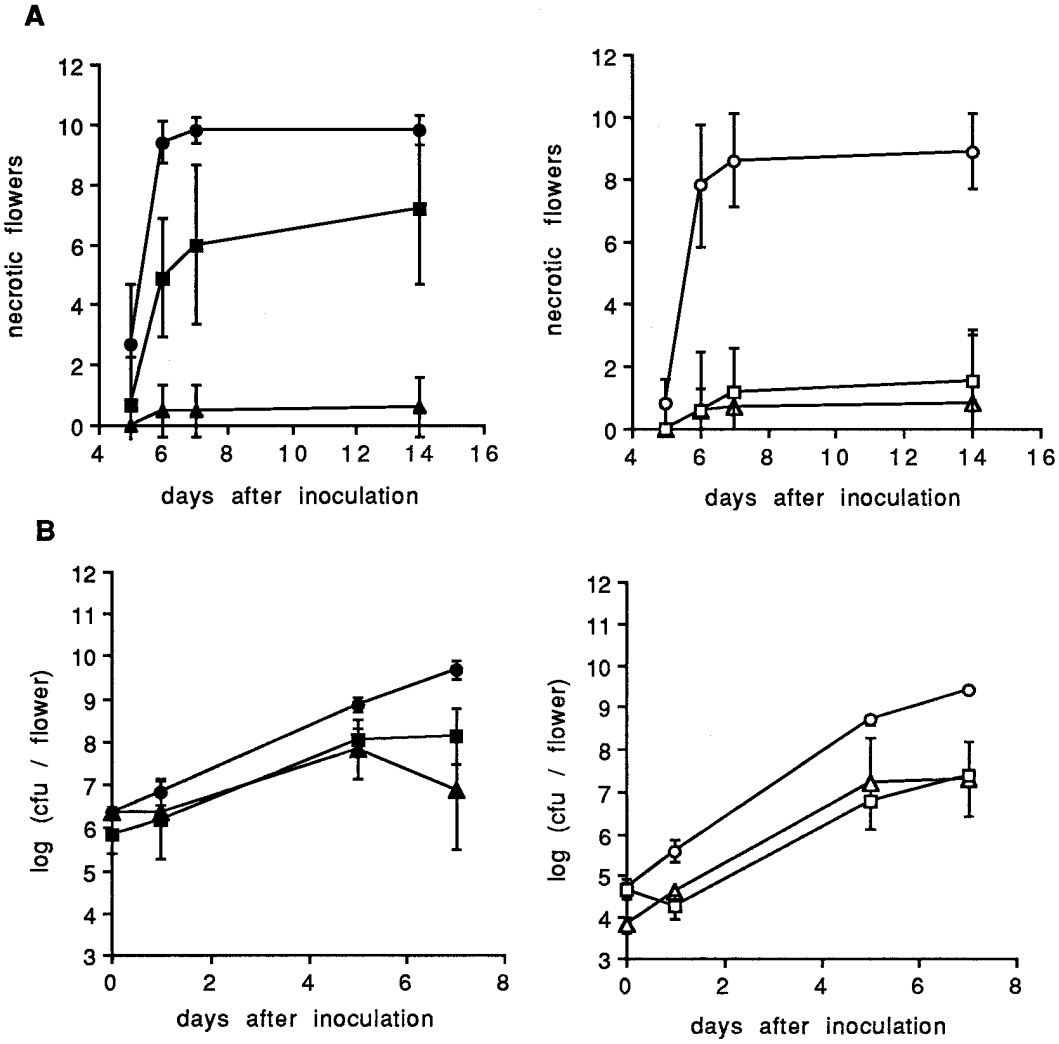

Fig. 6. Pathogenicity of wild-type strain (circles) and its derivatives harboring the mutation *dfoA-61* (squares) or *foxR-17* (triangles) on apple flowers. Details in Materials and Methods. Data shown correspond to the assay carried out in 1994. Timing of disease expression fluctuated but similar results were observed in 1995. **A,** Curves correspond to mean values calculated for 10 branches, obtained over time for each strain and each inoculum (filled symbols: 10^8 CFU/ml; open symbols: 10^6 CFU/ml). Standard deviations are indicated. **B,** Concurrent enumeration of bacterial populations was reported for each inoculum. Curves correspond to mean values obtained for each strain in six independant assays. Standard deviations are indicated.

to interfere with DFO. One event that can account for this situation is the oxidative burst. Indeed, situations of incompatibility and compatibility, the latter to a lesser extent than the former, generate reactive oxygen species that are responsible for membrane damage to plant cells and that can act as antimicrobial compounds (Low and Merida 1996). Concerning *E. amylovora*, it has been shown that harpin, elicitor of the HR and pathogenicity determinant, encoded by *hrpN* gene, elicits the production of active oxygen species from plant cells (Baker et al. 1993). DFO, by inhibiting the generation of OH°, could thus protect bacterial cells, preventing them from being the target of oxidative damage. The protection of pure culture of the wild-type strain and its mutants in the presence of physiological amounts of hydrogen peroxide by exogenous DFO, only when nonsaturated in iron, supports this interpretation. Interestingly, among the iron chelators tested in the assay, DFO appeared to ensure the higher level of protection against oxidative killing. The reduced ability of the *dfoA* mutant to induce electrolyte leakage can thus be explained by a transient decrease of bacterial population resulting from the oxidative burst. Interestingly, exopolysaccharides from pathogens have been shown to act as antioxidant scavengers (Miller and Britigan 1997; Kirali et al. 1997). Given that *E. amylovora* exopolysaccharide negative mutants are not changed in their ability to induce electrolyte leakage (Brisset and Paulin 1991), it is likely that, in this case, exopolysaccharides play no important role in protecting bacterial cells against oxidative killing. In contrast, the role of DFO would be essential. However, a second event may also occur. DFO could act as a substrate of plant cell wall peroxidases in the presence of H_2O_2, and then generate radical nitroxides, which could contribute in turn to membrane damage. At a critical concentration of bacteria, DFO could therefore enhance the oxidative stress induced by harpin, thus resulting in an overall increase of electrolyte leakage. It can be relevant in this respect to point out that both mentioned roles of DFO are known to occur at different concentration levels: low concentrations (1 to 10 µM range) leading to a protective effect, higher concentrations (100 µM range in presence of peroxidases) leading to a deleterious effect. As *E. amylovora* has the ability to punctually control the synthesis of DFO in intercellular spaces of plant tissues, it is conceivable that both effects occur in turn during the course of pathogenesis. These results also suggest that the level of saturation of DFO in iron is likely to be important during the infective process, which leads to speculation on the existence of a plant response modulating iron availability.

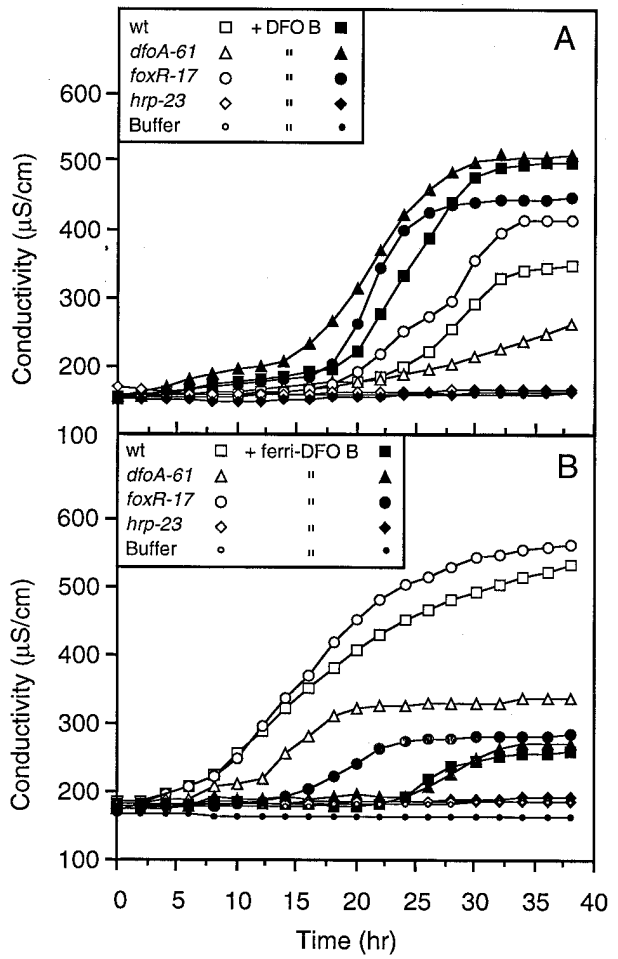

Fig. 7. Electrolyte leakage induced by wild-type strain and its mutant derivatives in nonhost (tobacco) leaf tissues. Details in Materials and Methods. Inoculum was **(A)** 10^8 CFU/ml and **(B)** 4×10^8 CFU/ml. Strain harboring mutation *hrp-23* and buffer were used as negative controls.

MATERIALS AND METHODS

Bacterial strains and microbiological techniques.

Strain CFBP 1430 is a natural isolate of *E. amylovora* (Paulin and Samson 1973). The DFO-deficient (VD61) and the ferrioxamine transport (VD17) mutants harboring mutations *dfo-61* (*dfoA::*Mu*d*IIpR13) and *foxR-17* (*foxR::* Mu*d*IIpR13) (Cm^r), respectively, derive from strain CFBP 1430 (Kachadourian et al. 1996). Mutant PMV6023 harbors the mutation *hrp-23::*Mu*d*IIpR13 (Barny et al. 1990). *E. coli* host strains were JM109, C600 for maintenance of plasmid pRK2013 and RW193 (F^- *entA thi trpE proC leuB lacY metl xyl galK ara rpsL azi tsx supE*). The rich medium was L broth (Miller 1972). Tris medium (Franza et al. 1991) was used to provide iron-limited conditions; it was supplemented with EDDHA or 2,2′-dipyridyl at a final concentration of 50 µM to chelate contaminating iron. It was supplemented with $FeCl_3$ at a final concentration of 20 µM for iron-replete conditions. For bacterial growth under various conditions of iron, an inoculum from an overnight culture in L broth was diluted in the medium to be tested to give an optical density at 600 nm of 0.01.

Table 1. Protective effect of by desferrioxamine (DFO) B on *Erwinia amylovora* cells exposed to H_2O_2[a]

Drugs added to the bacterial suspension	Strain survival (%)		
	Wild type	*dfoA-61*	*hrp-23*
None	100	100	100
H_2O_2	<0.4	<0.4	<0.4
H_2O_2 + DFO B	75 (25)	70 (30)	75 (25)
H_2O_2 + ferri-DFO B	<0.4	<0.4	<0.4
H_2O_2 + ferrozine	10 (5)	12 (5)	7 (5)
H_2O_2 + 2,2′-dipyridyl	1.6 (0.5)	1.7 (0.5)	1.4 (0.5)

[a] Survival of *E. amylovora* cells exposed to 1 mM H_20_2 in the presence of iron chelators (50 µM) was determined in three independent platings. Results are mean values of percentage of survivors relative to control obtained for each strain and condition, in three independent experiments. Numbers in parentheses indicate standard deviations.

Cultures were grown aerobically. Siderophore detection and cross-feeding assay were performed as described earlier (Kachadourian et al. 1996). For pathogenicity and H_2O_2 killing assays, the inoculum consisted of a bacterial suspension in distilled water made from a culture grown on King's B agar medium (King et al. 1954) for 24 h, adjusted to indicated concentrations. Bacterial suspensions, ranging from 10^4 to 10^5 CFU/ml, exposed to H_2O_2 (Sigma, St. Louis, MO), at indicated concentrations, were kept with continuous shaking. Viable counts were determined by plating serial dilutions on L agar medium. Glucose (2 g per liter) was used as carbon source. When necessary, antibiotics and nicotinic acid were used at concentrations indicated previously (Kachadourian et al. 1996). Bacterial cultures were kept at 28°C. DFO B (Desferal; Ciba Geigy, Basel Switzerland) and ferrozine (Sigma) were used at indicated concentrations. Formation of the ferric complex of DFO B (M:L ratio = 1:1) in H_2O was estimated spectrophotometrically, the absorbance maximum being at 432 nm.

Molecular biological methods.

The CFBP 1430 genomic library constructed in vector pLA2917 and general DNA techniques were described by Barny et al. (1990). Genomic sequences were determined on both strands by the dideoxynucleotide chain-termination method as described previously (Mahé et al. 1995) and analyzed with the Genetic Computer Group (GCG, Madison, WI) program. The sequence of the *E. amylovora foxR* gene has been submitted to the GenBank/EMBL data bank as accession number AJ223062. Total RNA was isolated from *E. amylovora* cells by the method of Aiba et al. (1981). Northern blot analysis was carried out as described previously (Expert et al. 1992).

Pathogenicity assays.

Apple seedlings were open-pollinated *Malus domestica* cv. Evereste. They were grown from seeds in the greenhouse after breaking the dormancy with a treatment at 4°C for 40 days, under high moisture conditions. Actively growing seedlings were used at the 8- to 10-leaf stage. The tip of the youngest expanding leaf was cut with scissors dipped into the bacterial suspension to be tested. Extension of symptoms on the midrib and petiole was scored daily; a symptom was considered to be systemic when the necrosis reached the stem. In each assay, 15 plants were inoculated by each strain and assays were carried out in triplicate. Apple flowers were collected from *Malus domestica* cv. Golden Delicious trees, grown in the orchard. Branches bearing 10 blossoms were potted into buckets containing gravel and water and maintained in the greenhouse at 25°C under 90% relative humidity to allow bud development. Under these conditions, healthy flowers were able to form fruits in 3 weeks. For inoculation, two flowers were kept per flower cluster. They were manually fertilized with compatible pollen to allow a normal fruit-set. The bases of pistils of open flowers were spotted with 10 µl of the bacterial suspension to be tested. Two concentrations of inoculum were used per strain. For each condition, 200 single flowers were inoculated. Progression of symptoms was scored for one of the two inoculated flowers and bacterial population was assessed from the second inoculated flower of the same blossom. At each indicated time, viable counts were made from crushed tissues, divided in three batches of five flowers, after plating on L agar medium containing the appropriate antibiotics. Assays were performed during two consecutive springs.

Electrolyte loss determination.

Leaves were collected from seedlings of *Nicotiana tabacum* cv. Xanthi. Electrolyte loss was determined as described previously (Brisset and Paulin 1991), except that the procedure was miniaturized, with automatic measurements. In brief, leaf disks (5 mm diameter) were vacuum infiltrated with a bacterial suspension in appropriate assay medium (0.5 mM MES [morpholineethanesulfonic acid], 0.5 mM $CaCl_2$, pH 6), containing 100 µM DFO B or ferri-DFO B when required. Disks were then blotted dry for 30 min and incubated in vials with assay medium containing 100 µM DFO B or ferri-DFO B when required, under light conditions, at 25°C, with continuous stirring. Three assays of 6 disks in 2.5 ml of medium per treatment were carried out. Conductivity probes (Tacussel XE 120) were permanently immersed in each vial and connected to a computer-monitored conductivimeter. Measurements were performed every 2 h for 45 h. Each experiment was performed at least twice.

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